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# Headspace and direct immersion solid phase microextraction procedures for selenite determination in urine, saliva and milk by gas chromatography mass spectrometry

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ABSTRACT

Two solid phase microextraction modes were investigated and compared for their performance on the determination of selenites in various biological liquids like human urine and saliva and various types of milk. Using sodium tetraethylborate ($NaBEt_4$) as ethylating reagent, selenites are converted *in situ* to volatile diethylselenides (DESe) in aqueous medium. The derivative is collected *in situ* by solid phase microextraction (SPME) using a silica fiber coated with poly(dimethylsiloxane) (PDMS) either from the headspace (HS-SPME) or directly from the liquid phase (LP-SPME) and finally determined by capillary GC/MS. Under optimum conditions of SPME, the GC separation was also optimized. Between the two examined microextraction techniques, direct immersion of the PDMS fiber in the liquid phase was proved less satisfactory. In contrast, the headspace procedure appears to be more efficient. The quantification of selenites was achieved in SIM mode with good analytical performance. A non-fat milk powder certified reference material was analyzed to evaluate the accuracy of the method. The overall precision of the method was ranged between 6.2% and 9.7%. Detection limits achieved were 0.05 $\mu g\,L^{-1}$ for human urine, 0.08 $\mu g\,L^{-1}$ for saliva and 0.03–0.06 $\mu g\,L^{-1}$ in various milk matrices.



## 1. Introduction

Selenium is known to be toxic at higher concentration levels, causing negative health effects and, in contrast, a necessary trace element at low levels, depending on its various chemical forms and level of intake [1–3]. In recent years the identification and quantification of selenium species has gained increasing importance due to its role in prevention and chemoprevention of several diseases including cancer [3–5]. Selenium can exist in inorganic forms or as organoselenium compounds. The most important of these are selenide ($Se^{-2}$), selenite ($Se^{+4}$), selenate ($Se^{+6}$) dimethylselenide (DMSe), dimethyldiselenide (DMDSe), selenourea and selenoaminoacids. DMSe and DMDSe are considered to be 500 times less toxic than selenite and are found in environmental and biological samples due to biomethylation processes [6–8]. Plants and animals metabolize inorganic or organic species with bacteria and microorganisms and synthesize selenoproteins [1]. Selenium is found in common food sources for general population therefore, it can be easily absorbed by human and other organisms. It is also widely used in various industrial applications, such as semiconductors, ceramic and glass products, etc. [9].

Selenite ions ($Se^{+4}$) can be transformed to diethylselenide species (DESe), a more volatile and less polar compound, by suitable ethylation reagents. Several modern preconcentration methods were reported for the determination of selenium species [6,10]. Volatile diethylselenide compounds can be collected from sample matrices by headspace or liquid phase microextraction. SPME is used to achieve a dynamic equilibrium between the gas or liquid phase and the fiber coating phase. SPME procedures were studied by Guidotti [7] for selenite determination in tap waters. The derivatized species could be adsorbed efficiently onto a polydimethylsiloxane (PDMS) fiber. A significant drawback is the difficulty to automate the procedure. However nowadays an automated SPME manifold is commercially available on a 96-well plate format [11,12]. Headspace SPME is preferred for gas chromatographic analysis of biological fluids [13].

Selenium can also be measured efficiently by hydride generation, atomic absorption or fluorescence spectrometry [14,15]. On the other hand analytical techniques which are employed for selenium speciation are usually hyphenated techniques, including capillary electrophoresis [16,17] gas or liquid chromatography coupled to one or more of several commercially available detectors [4,18]. Gas chromatography is a sensitive and powerful speciation technique and has so far been used for organoselenium compounds in variable matrices like yeast, garlic, food supplements, biological matrices, etc. The most frequently used modern techniques

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**Table 1**
Optimum operating conditions for GC-MS.

| Operating conditions | Value |
|---|---|
| *Column DB-5MS* | 30 m × 0.25 mm i.d. × 0.10 μm |
| *Injection mode* | Splitless |
| *Injector port temperature* | 230 °C |
| *Oven program/ramp* | 40 °C (1 min) to 250 °C (0 min), 45 °C min$^{-1}$ |
| *Carrier gas; flow-rate* | Helium; 4.2 mL min$^{-1}$ |
| *Transfer line temperature* | 250 °C |
| *MS model* | 5973 quadrupole mass-selective detector |
| *SIM measured ions (m/z)* | 78, 80, 82 (selenium)<br>107, 110, 111 (ethylselenide)<br>136, 138, 139, 140 (diethylselenide) |
| *Electron ionization* | 70 keV |
| *SIM dwell time* | 50 ms |
| *MS quad/source temperature* | 150 °C/230 °C |

for such purpose are GC/MS [7,19,20], GC/AED [11], GC/MIP-AED [8,9,18] and ICP/MS [6,9,21–26]. Selenium levels in biological tissues and liquids of adult population were studied by Raghunath et al. [2] and metal speciation in biological fluids was investigated by Das et al. [27]. Selenium compounds in urine are determined by different techniques. For example, GC/MS [19], and HG/LT-GC/ICP-MS [26] were applied to human urine after fish consumption. Also, HS-SPME-GC/ICP/MS [25] and ID-GC/MS were employed for determination of selenium in urine using various derivatizing reagents [20].

Despite of the fact that biological liquids are very important for toxicological and nutritional purposes, as far as we know, for several of them (e.g. saliva) no previous reports are found for selenite determination using the SPME technique. Accordingly, in this paper, a method was developed and optimized for selenite ($Se^{+4}$) determination by GC/MS in biological liquids including urine, saliva and milk. In addition, to further optimize the developed method two different SPME approaches were compared, the headspace (HS-SPME) and the liquid phase (LP-SPME) solid phase microextraction.

## 2. Experimental

### 2.1. Instrumentation

The chromatographic analysis was performed with an Agilent 6873K gas chromatograph with a capillary column DB-5MS (30 m length, 0.25 mm id, 0.1 μm film, Supelco Co.), coupled to an Agilent Quadrupole Mass Spectrometric Detector 5973 and operated through the Agilent ChemStation software. Helium was used as the carrier gas. All GC and MS operating conditions are summarized in Table 1. Manual SPME fiber holder (57330-U) and polymer coated fibers (57342-U) were purchased from Supelco (Deisenhofen, Germany). The fibers were coated with a 100 μm film of PDMS and used for sampling of ethylated selenite in 15-mL glass vials.

### 2.2. Reagents and materials

Selenite intermediate standard solutions with concentrations from 10 mg L$^{-1}$ down to 100 μg L$^{-1}$ (as Se) were prepared weekly from a stock solution of selenite ($Na_2SeO_3$) in 0.5 mol L$^{-1}$ $HNO_3$ (Merck, Darmstadt, Germany) containing 1000 mg L$^{-1}$ Se. All stock, diluted and working solutions were prepared by diluting with Milli-Q ultrapure water (18.2 MΩ cm, 25 °C, Millipore, USA). The solutions were stored at 4 °C in the dark and were sealed with parafilm. Working standard solutions of lower concentrations down to 0.05 μg L$^{-1}$ (as Se) were prepared daily, immediately before use.

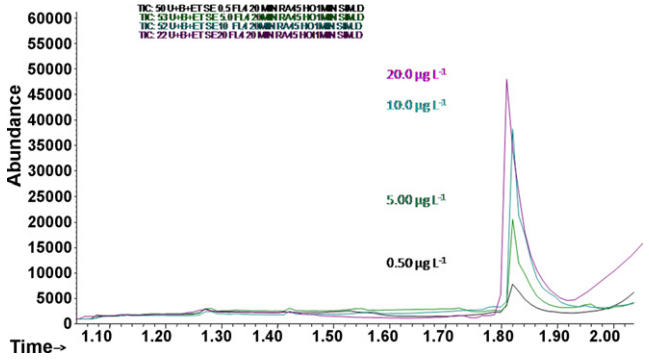


**Fig. 1.** Typical chromatograms in SIM mode obtained from the determination of selenite in human urine spiked with 0.50, 5.00, 10.0, 20.0 μg L$^{-1}$ as Se.

Sodium tetraethylborate was purchased from (99.5%, Sigma–Aldrich Newburgport, MA, USA). A 20% (w/v) solution was freshly prepared by dissolving 0.20 g $NaBEt_4$ in 1.0 mL Milli-Q water, and was stored at 4 °C. Buffer solution with pH value of 5.2 ± 0.1 and an ionic strength ca. 0.4 mol L$^{-1}$ was prepared by mixing appropriate amounts of sodium acetate (2 mol L$^{-1}$) and acetic acid (2 mol L$^{-1}$) in a final volume of 1 L. The method performance was validated with the standard reference material NIST 1549 non-fat milk powder.

### 2.3. Headspace and direct immersion SPME

A volume of 5.0 mL of the above described buffer solution ($CH_3COOH$–$CH_3COONa$, pH 5.2) and 5.0 mL of Milli-Q water or standard solution or biological liquid (urine or saliva) was transferred into 15-mL glass vial and closed with PTFE-coated septum. The alkylation–extraction process which was applied for milk samples includes precisely weighing 0.100 g milk powder (skimmed or full) and equilibration with the acetate buffer solution in a final volume 10 mL. The best results were received with 0.100 g milk powder. For the standard addition procedure, suitable portions of a 100 μg L$^{-1}$ selenite standard solution was added to the buffered sample, followed by addition of 50 μL of 20% (m/v) $NaBEt_4$. The vial was closed, the mixture was stirred at 900 rpm for 5 min, sonicated in supersonic vibration for 5 min for further mixing, and finally centrifuged at 3500 rpm for 3 min.

In HS-SPME mode the fiber was placed just 7 mm over the surface while in LP-SPME the fiber was immersed in the solution. In both modes the temperature of the solution was 25 °C after optimization. The fiber was removed from the vial and immediately transferred into the GC injection port at 230 °C and left there for complete desorption (4 min). The peak of selenite as diethylselenide appears in 1.821 min (Fig. 1).

## 3. Results and discussion

Optimization of SPME and GC conditions was performed in the scan mode and quantification in selected ion monitoring (SIM) mode using ions with *m/z* values as listed in Table 1. Because the ethylation and extraction processes are simultaneous, they were optimized separately for the headspace SPME procedure and for the direct immersion SPME, as it is described below, analyzing buffered aqueous solutions in triplicate. The optimized conditions were then applied to the analysis of real samples.

### 3.1. Optimization of HS-SPME

The acidity of the solution is probably the most important parameter which defines the efficiency of the derivatization reac-

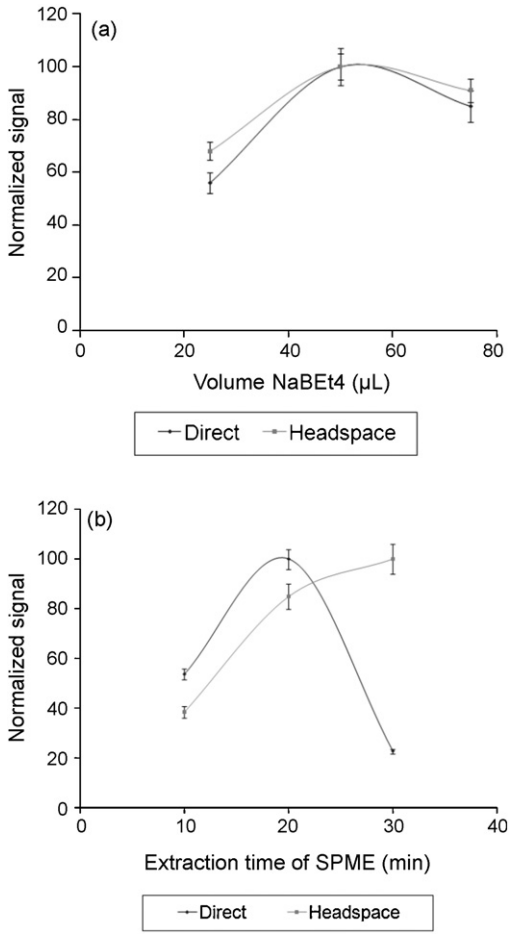


**Fig. 2.** Optimization of HS-SPME or LP-SPME with $NaBEt_4$: effect of (a) volume of derivatization reagent 20% (m/v) $NaBEt_4$ and (b) the extraction time on selenite normalized signal.

tion. The effect of decreasing acidity was studied in the range of pH 3.2–7.2 respectively. The optimum value was found to be $5.2 \pm 0.1$, which is in accordance with previously reported values for ethylation by tetraethylborate [3].

The desorption temperature applied in the sample injection port was also optimized. It is critical to ensure complete desorption of the analytes from PDMS fiber to inlet and then to GC column. Desorption temperatures 210 °C, 230 °C and 250 °C were studied, resulting in not significant differences in peak area and eventually 230 °C was selected.

The volume of derivatization reagent was also investigated. The effect of the amount of $NaBEt_4$ is significant since the volume of alkylation reagent must be sufficient to ethylate all the amount of selenite in the sample. On the other hand use of the reagent in excess leads to the formation of other products during alkylation (e.g. boroxine), which elute and may overlap or affect the analyte peak. The effect of the amount of $NaBEt_4$ was examined by adding different amounts (25 μL, 50 μL, 75 μL) of a 20% (m/v) solution of $NaBEt_4$ to a spiked solution. A spiked solution containing 10 mL buffer solution pH $5.2 \pm 0.1$ and 200 μg $L^{-1}$ selenite ($Se^{+4}$) was prepared in 15-mL glass vial and closed with PTFE-coated septum. As it is illustrated in Fig. 2a the best results were obtained using 50 μL of the derivatization reagent.

The extraction time is another critical parameter for the overall sensitivity of the method. The duration of HS-SPME defines the efficiency to collect selenite from the solution. The effect of increasing the extraction time (10 min, 20 min and 30 min) was studied at 25 °C and the results are illustrated in Fig. 2b. Ten minutes was a very short time for efficient extraction (<60%), while after 20 min most selenite (in the form of diethylselenide) was collected to PDMS fiber in SPME. The normalized signal increased after 30 min but the increase is not linear. In conclusion, 20 min headspace sampling time was selected for all further analysis.

The temperature during of HS-SPME was varied between 25 °C and 45 °C, and eventually 25 °C was found to give the highest signal to noise ratio (higher sensitivity). At this temperature, the derivatization was more efficient and clear peak of selenite was taken without overlapping by other compounds like boroxine, which is formed by side reaction. A further problem from the presence of co-eluting boroxine peak tail arise from the fact that it has a molecular ion $m/z$ 81 ($B_3O_3$ 81 g $mol^{-1}$) close to that of the isotopic pattern of selenium, and a mass spectrum which has similarities to that of derivatized selenides. In this case, ion $m/z$ 168 of triethylboroxine and the boroxine isotopic pattern can be helpful to distinguish from selenite.

### 3.2. Optimization of LP-SPME

The question was whether the alkylated selenite species could be extracted more efficiently by immersing the fiber of PDMS into the urine matrix. Thus the effects of the extraction time and extraction temperature during liquid phase SPME of selenite were investigated. Keeping the extraction temperature at 25 °C the effect on the normalized signal was examined after 10 min, 20 min and 30 min extraction time and at 20 min extraction time the higher signals were obtained (Fig. 2b). Next, using 20 min as extraction time, the extraction temperature was investigated at 25 °C, 45 °C and 65 °C respectively and the results proved that the higher extraction was achieved at the lower temperature of 25 °C.

### 3.3. Optimization of GC-MS analysis

Using the above found optimum or selected values for HS-SPME the critical parameters of the gas chromatography were optimized. The splitless mode in the GC injection port was employed using a specific liner (narrow bore 0.75 mm i.d).

Selenite after the derivatization with sodium tetraethylborate was converted to diethylselenide which is a volatile compound and therefore a low initial temperature was chosen (40 °C). At this temperature the hold time was varied between 1 min and 4 min and the results are presented in Fig. 3a. Optimum hold at initial temperature was found at 1 min (the normalized signal increased rapidly and sharper peaks are taken). The final temperature of the program was 250 °C and the transfer line between column and MS detector was heated at 250 °C.

The temperature ramp from 40 °C to 250 °C was also investigated and optimized. At 45 °C $min^{-1}$ the peak tailing was minimised and no carryover effects from other compounds could be observed (Fig. 3b). The total time of chromatogram is less than 6 min.

Flow rate is a critical effect of optimization of GC. The helium flow conditions were selected under constant pressure and variable flow rate of helium. Three different flow rates i.e. 2.2 mL $min^{-1}$ at 17.4 psi, 4.2 mL $min^{-1}$ at 29.73 psi and 6.2 mL $min^{-1}$ at 39.3 psi, were studied respectively and the optimum flow rate in terms of normalized signal (higher peak area, sharper peaks and clearly chromatograph without overlapping), was obtained at 4.2 mL $min^{-1}$, which was used for subsequent work.

### 3.4. Calibration studies

Comparative calibration curves for selenite (in the form of diethylselenide after ethylation) and HS-SPME extraction or direct LP-SPME were obtained. The slope of the regression equation for HS-SPME was $y = (444 \pm 6.6)\ 10^2\ c_{Se}$ and the correlation coefficient

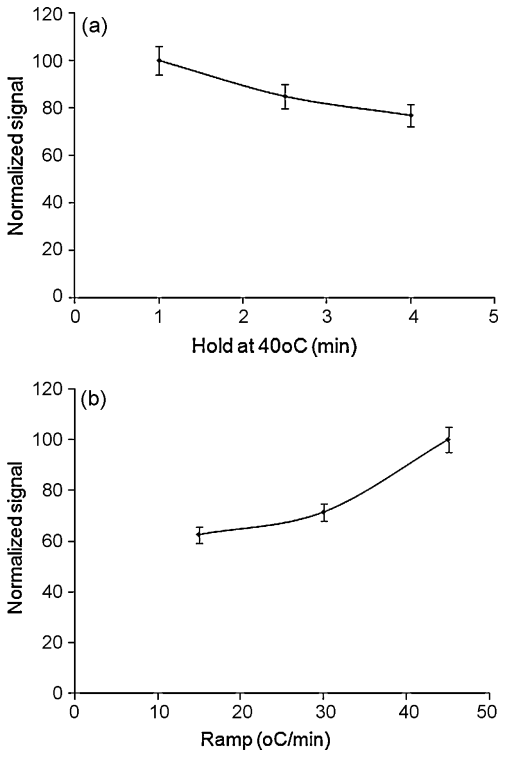


**Fig. 3.** Optimization of GC-MS with $NaBEt_4$ and SPME. Effect of (a) hold time at initial temperature 40 °C and (b) ramp from 40 °C to 250 °C on selenite normalized signal.

$r = 0.9996$ while for LP-SPME it was $y = (78 \pm 6.6)\,10^2\,c_{Se}$ and the correlation coefficient $r = 0.9946$. With HS-SPME improved sensitivity was achieved as compared to the LP-SPME, therefore HS-SPME is recommended for determination of selenite.

After optimization, the method was applied to various samples in order to check its performance. Different calibration or standard addition curves were taken under optimum conditions for several types of biological samples like urine and saliva (Fig. 4a). Also, standard addition curves were taken under optimum conditions for different milk products like milk powder (full or skimmed) and fresh pasteurized milk (Fig. 4b). Blank matrices of urine and milk were prepared by addition of buffer and ethylation reagent and final degassing.

The comparison of standard addition curves shows that improved sensitivity is obtained in urine matrix. The calibration curves with the highest sensitivity were obtained for water which has no matrix effect followed by those obtained for human urine samples. Selenites can be measured efficiently in buffered urine samples as in water samples.

The standard addition curve for skimmed milk powder had better sensitivity (slope) in comparison to the full milk powder or full fresh pasteurized milk. A possible reason is that skimmed milk powder was non-fat and selenite could react quantitatively. On the other hand the lowest sensitivity obtained in saliva samples may be attributed to the presence of proteins which may form complexes with selenite and alter the gas–liquid equilibrium. The analytical performance characteristics of the method when applied to the particular biological matrices are discussed in details in the following section. The detection limit ($c_L$) was calculated based on the IUPAC definition of $c_L = 3s/S$ where ($s$) is the standard deviation of a series of ten blank solutions and ($S$) the slope of the calibration curve.

Among the most common inorganic selenium species, selenites and selenates are more toxic and more biochemically functional forms [10]. In preliminary experiments to examine the possibility

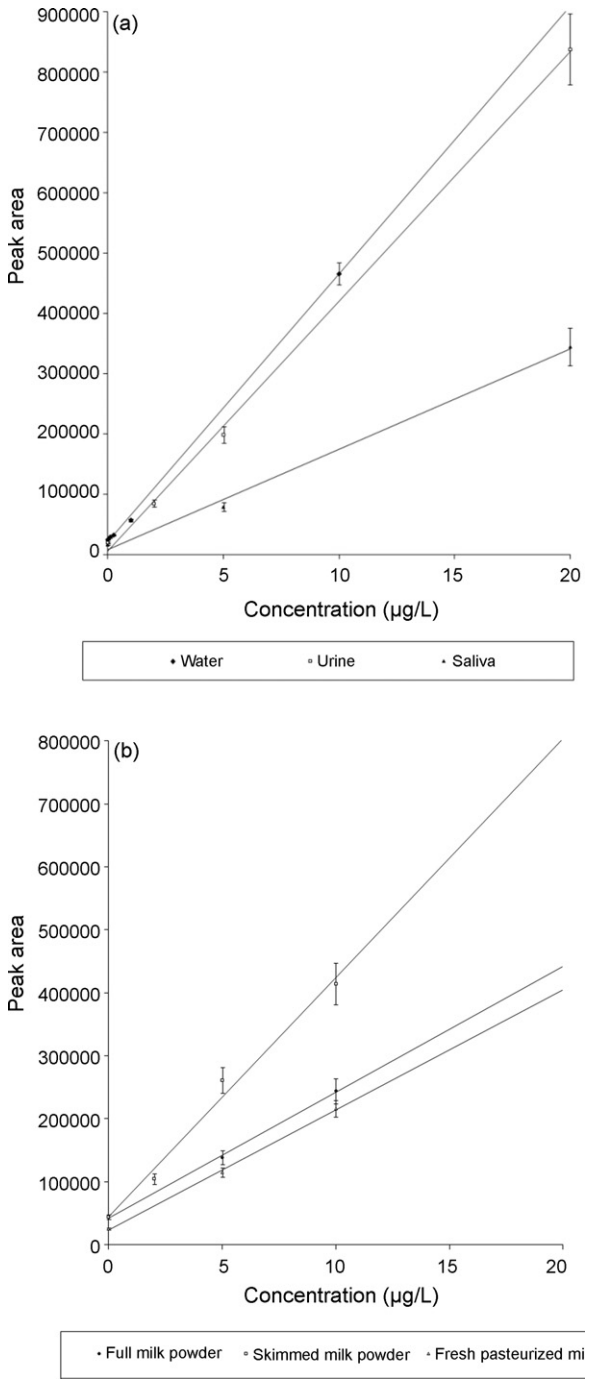


**Fig. 4.** Comparative calibration curves for selenite as diethylselenide after ethylation with $NaBEt_4$ and HS-SPME extraction. (a) Aqueous standards, human urine, human saliva and (b) milk powder (full or skimmed) and fresh pasteurized milk samples.

of selenates ($Se^{+6}$) derivatization with the developed method the results were not satisfactory. A small peak, not always measurable, was observed in the chromatogram just after that of selenite, however this could not be further confirmed. It is known form previous reports of Guidotti et al. [7,29] that selenates are not derivatized as selenites in SPME and GC separations. In order to perform a speciation between them usually ion chromatography or hydride generation atomic absorption spectrometry (HGAAS) is applied. In the HGAAS the two species are sequentially determined by differential calculation. Selenites are determined before and selenates after strong acidification by hydrochloric acid [30]. However, this

**Table 2**
The analytical performance characteristics of the proposed method.

| | Urine | Saliva | Milk powder | Skimmed milk powder | Pasteurized milk |
|---|---|---|---|---|---|
| Linearity ($\mu g\,L^{-1}$) | 0.10–20 | 0.24–20 | 0.15–40 | 0.09–10 | 0.18–10 |
| Corr. Coefficent. ($r$) | 0.9995 | 0.9981 | 0.9995 | 0.9935 | 0.9994 |
| Sensitivity | $(414 \pm 9.4) \times 10^2$ | $(166 \pm 10) \times 10^2$ | $(200 \pm 6.2) \times 10^2$ | $(380 \pm 30) \times 10^2$ | $(190 \pm 6.5) \times 10^2$ |
| Recovery (%)[a] | $96.4 \pm 3.2$ | $90.3 \pm 2.1$ | $103.4 \pm 5.1$ | $101.2 \pm 11.1$ | $97.1 \pm 2.3$ |
| LOD ($c_L$, $\mu g\,L^{-1}$) | 0.05 | 0.08 | 0.05 | 0.03 | 0.06 |
| RSD ($s_r$, %)[a] | 6.3 | 7.8 | 8.5 | 6.2 | 9.7 |

[a] Recovery and precision calculated for $n = 5$ spikes of $\gamma = 2.5\ \mu g\,L^{-1}$.

approach could not be applied in this work, since the strong acidic medium did not allow the ethylation process which requires mild acidic conditions.

### 3.5. Method performance in biological matrices

#### 3.5.1. Urine samples

Selenium is found in many organisms and can be detected in human biological samples. The presence of selenium in urine and saliva is normal and mainly due to Se-containing food consumption [28]. The results of the calibration studies from determination of selenium as diethylselenide using the proposed method are listed in Table 2. The limit of detection was $0.05\ \mu g\,L^{-1}$, the precision (RSD) was 6.3% and the recovery was $96.4 \pm 3.2\%$ respectively. It is worth to mention that in recent selenium speciation studies by SPME-GC-AED [31] the detection limit for selenites was $7.3\ ng\,L^{-1}$ and the precision was 6.1% as calculated from the analysis of spiked tap water samples, using the same derivatization reagent and a sensitive atomic emission detector.

#### 3.5.2. Saliva samples

A pooled matrix of saliva sample was prepared which after pH adjustment and addition of alkylation reagent was stirred and degassed. Finally standard addition was done to the obtained blank matrix. The linearity in saliva samples ranged between $0.24\ \mu g\,L^{-1}$ and $20.0\ \mu g\,L^{-1}$ (Table 2). The limit of detection was $0.08\ \mu g\,L^{-1}$, the RSD was 7.8% and the recovery was $90.3 \pm 2.1\%$.

#### 3.5.3. Milk samples

The accuracy of the developed HS-SPME method was estimated using the NIST-1549 (non-fat milk powder) certified reference material. The certified value was $0.11 \pm 0.01\ mg\,kg^{-1}$ and the found value was $0.107 \pm 0.002\ mg\,kg^{-1}$ (recovery $97.2 \pm 1.9\%$). Three different milk products, namely full milk powder, skimmed milk powder and full fresh pasteurized liquid milk were examined for the method performance. It was observed that the behavior was almost the same for full milk powder and full pasteurized liquid milk. Limits of detection for Se were $0.05\ \mu g\,L^{-1}$ for full milk powder and $0.06\ \mu g\,L^{-1}$ for full fresh pasteurized liquid milk respectively. The RSD was 8.5% and 9.7%, while the recovery ranged between $103.4 \pm 5.1\%$ and $97.1 \pm 2.3\%$ respectively (Table 2). It was proved that the method performance in full fat matrices is similar while the sensitivity is increased in skimmed milk powder and the limit of detection is lower.

## 4. Conclusions

Fast screening methods for the determination of selenites in biological matrices are of constant interest. Headspace SPME after suitable derivatization with tetraethylborates appears to be more efficient separation and preconcetration system for selenite determination in various biological matrices as compared to the direct liquid phase SPME. The method was successfully applied to the measurement of selenite in urine, saliva, milk powder (with full fat or skimmed) and fresh pasteurized milk. The results showed that in urine samples and the non-fat milk powder the performance of the method is better, while in presence of increased lipid or protein concentration the overall performance is deteriorated. The concentration found from the analysis of certified reference material is in good agreement with the reference value and the overall analytical performance of the proposed method makes it useful as a routine method for the determination of selenite in the examined biological matrices.

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